International Journal of Biology Research
ISSN: 2455-6548, Impact Factor: RJIF 5.22
www.biotechjournals.com
Volume 1; Issue 5; November 2016; Page No. 37-42

Investigation on phytochemical content and antioxidant activity of locally grown garlic (*Allium sativum* L.) in Bangladesh

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Abstract

Background: Garlic (*Allium sativum* L.) belonging to the Alliaceae family has played important dietary and medicinal roles throughout the history.

Objectives: The present study was undertaken to investigate the phytochemical content as well as antioxidant activity of local variety garlic extract using different solvent systems in Bangladesh.

Methods: Five different types of extract were prepared using methanol, ethanol, acetone, chloroform and petroleum ether as solvent. We performed the phytochemical screening and determined the total phenolic, flavonoid, flavonol and proanthocyanidin content of the extracts using standard procedures. Several *in vitro* assay models were employed to investigate the antioxidant activity.

Results: Out of the five extracts, the acetone extract of local variety possessed the highest content of phenolics (110.76±1.9mg of gallic acid equivalent/gm of dry extract)), flavonoids (43.32±2.7mg of catechin equivalent/gm of dry extract), flavonols (15.31±2.3mg of quercetin equivalent/gm of dry extract)) and proanthocyanidins (8.54±0.5 mg of catechin equivalent/gm of dry extract). The radical scavenging activity, the total antioxidant capacity as well as the reducing power of the extracts increased with the increase of concentration. In total antioxidant capacity test and ferric reducing antioxidant power assay acetone extract of local variety showed the highest activity. In DPPH, ABTS, hydroxyl radical scavenging assay and lipid peroxidation inhibition assay strong antioxidant activities were observed by each extract in which acetone extract of local variety was found to be the best one with the IC_{50} values (in µg/ml) of 5.1±0.9 (DPPH assay), 11.3±0.2 (ABTS assay), 15.7±0.6 (Hydroxyl radical assay) and 19.5±0.3 (Lipid peroxidation assay).

Conclusion: Findings of the present study suggested that local variety garlic of Bangladesh is a promising source of natural antioxidant and could have great importance as therapeutic agent in various diseased conditions. The study also recommended that acetone would be the preferable solvent for extracting polyphenols from garlic.

Keywords: garlic extract, phytochemical, antioxidant activity

1. Introduction

Imbalance between production and scavenging of free radicals and other reactive oxygen species (ROS), known as oxidative stress, is an important component of pathophysiology of countless human diseases like atherosclerosis, cancer, diabetes, cardiovascular disease, ageing and inflammatory diseases. All biological systems have antioxidant defense mechanism that protects against oxidative damages and repairs enzymes to remove damaged molecules. However, this natural antioxidant defense mechanism often fails; hence dietary intake of antioxidant is important. Currently, synthetic antioxidants such as butylated hydroxytoluene (BHT) are most commonly included in food products. However, the synthetic antioxidant BHT has been suspected of causing liver damage due to their toxic properties [1, 2]. Recently, medicinal plants have attracted particular attention in research due to their diverse pharmacological properties.

Garlic (*Allium sativum L.* family: Alliaceae) has played important dietary and medicinal roles throughout the ages. Several *in vitro* and *in vivo* investigations suggested many favorable effects of garlic and its preparations [3, 4]. A morphologically distinct variety of garlic is locally cultivated in Bangladesh, but there is no available scientific data regarding the pharmacological properties of this variety. Hence, the present study investigated the phytochemicals of locally grown garlic extract and determined its antioxidant activity.

2. Materials and Methods

2.1 Collection of plant material and authentication

For the present study, local variety garlic of Bangladesh was collected from the local area of Rajshahi (north-western part of Bangladesh) and authenticated by the Department of Botany, University of Rajshahi, Bangladesh.

2.2 Preparation of extract

The garlics were first washed with water to remove adhering dirt, chopped into small pieces and then shed dried. After complete drying, the entire portions were grinded into a coarse powder by a grinding machine and stored in an airtight container for further use. Five different solvents namely ethanol, methanol, chloroform, acetone and petroleum ether were used for extraction. For each solvent about 100gm of the powdered material was taken in separate clean, round bottomed glass bottle and soaked in 500 ml of solvent. The container with its content was sealed by cotton plug and aluminum foil and kept for a period of 15 days accompanying occasional shaking and stirring. The resulting extracts were filtered through Whatman No. 1 filter paper. Afterwards, the solvents were evaporated under reduced pressure at 39˚C using rotary evaporator. Finally, the residues were kept in small sterile bottles under refrigerated conditions until used. Thus, five types of extracts of garlic were obtained as Methanol Extract (MEG), Ethanol Extract (EEG), Chloroform Extract (CEG), Acetone Extract (AEG) and Petroleum ether Extract (PEG).

2.3 Chemicals and reagents

All the chemicals and reagents used throughout this investigation were of reagent grade.

2.4 Phytochemical screening of the extracts

Qualitative phytochemical tests were carried out to detect some bioactive components present in the extracts. The main bioactive groups (Polyphenols, Terpenoids, Steroids, Saponins, Tannin, Flavonoids, Alkaloids and Glycosides) were identified in each extract using different standard methods [5].

2.5 Estimation of total phenolics

Total phenolic contents of the extracts were determined by the modified Folin-Ciocalteu method described by Wolfe *et al.* [6]. An aliquot of the extracts/standard was mixed with 2 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 2 ml (75 g/l) of sodium carbonate. The test tubes were vortexed for 15 seconds and kept for 20 minutes at 25°C for color development. Absorbance was then measured at 760 nm using UV-spectrophotometer (Shimadzu, USA). Total phenolic contents were expressed in terms of gallic acid equivalent, GAE, mg of GA/g of dry extract.

2.6 Determination of total flavonoids

Total flavonoids were estimated using the method described by Ordonez *et al*. [7]. To 0.5 ml of samples/standard, 1.5 ml of methanol, 100 µl of 10% $AlCl_3$, 100 µl of 1M potassium acetate solution and 2.8 ml of distilled water were added. After 90 minutes of incubation at room temperature (RT), the absorbance was measured at 420 nm. Total flavonoid contents were expressed in terms of catechin equivalent, CAE, mg of CA/g of dry extract.

2.7 Determination of total flavonols

Total flavonols in the plant extracts were estimated using the method of Kumaran and Karunakaran [8]. To 2.0 ml of sample/standard, 2.0 ml of 2% $AlCl_3$ in ethanol and 3.0 ml (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 hours at 20°C. Extract/standard was evaluated at a final concentration of 0.1 mg/ml. Total content of flavonols was expressed in terms of quercetin equivalent, QUE, mg of QU/g of dry extract.

2.8 Determination of total proanthocyanidins

Determination of content of proanthocyanidins was based on the procedure reported by Sun *et al*. [9]. A volume of 0.5 ml of 0.1 mg/ml of extracts/standard solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 minutes. The absorbance was measured at 500 nm. Total content of proanthocyanidins was expressed in terms of catechin equivalent, CAE, mg of CA/g of dry extract.

2.9. Determination of Antioxidant Activity

2.9.1 Determination of total antioxidant capacity

Total antioxidant capacity (TAC) of samples/standard was determined by the method reported by Prieto *et al*. [10] with some modifications. 0.5 ml of samples/standard at different concentrations was mixed with 3 ml of reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate into the test tubes. The test tubes were incubated at 95°C for 10 minutes to complete the reaction. The absorbance was measured at 695 nm using a spectrophotometer against blank after cooling at RT. BHT was used as standard. A typical blank solution contained 3 ml of reaction mixture and the appropriate volume of the same solvent used for the samples/standard. Each tube was incubated at 95°C for 10 minutes and the absorbance was measured at 695 nm.

2.9.2 Determination of ferric reducing antioxidant capacity

The ferric reducing antioxidant capacity of samples/standard was evaluated by the method of Oyaizu [11]. 0.25 ml of samples/standard solution at different concentrations, 0.625 ml of potassium buffer (0.2 M) and 0.625 ml of 1% potassium ferricyanide $[K_3Fe\ (CN)_6]$ solution were added into the test tubes. The reaction mixture was incubated for 20 minutes at 50°C to complete the reaction. Then 0.625 ml of 10% TCA solution was added into the test tubes. The total mixture was centrifuged at 3000 rpm for 10 minutes. After that, 1.8 ml supernatant was withdrawn from the test tubes and was mixed with 1.8 ml of distilled water and 0.36 ml of 0.1% $FeCl_3$ solution. The absorbance of the solution was measured at 700 nm using a spectrophotometer against blank. A typical blank solution contained the same solution mixture without plant extracts/standard was also incubated under the same condition, and the absorbance of the blank solution was measured at 700 nm.

2.9.3 DPPH free radical scavenging assay

DPPH free radical scavenging activity was determined using the method described by Choi *et al*. [12]. A solution of 0.1 mM DPPH in methanol was prepared and 2.4 ml of this solution was mixed with 1.6 ml of extractives in methanol at different concentrations. The reaction mixture was vortexed thoroughly and left in the dark at RT for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517 nm. BHT was used as reference standard. Percentage DPPH radical scavenging activity was calculated by the following equation: $S\% = [(A_0 - A_1)/A_0)] \times 100$, where A_0 is the absorbance of the control without sample, and A_1 is the absorbance of the extractives/standard. Then percentage DPPH radical scavenging activity was plotted against concentration, and from the graph IC_{50} was calculated.

2.9.4 ABTS radical scavenging assay

The antioxidant capacity was estimated in terms of the

ABTS●+ radical scavenging activity following the procedure described by Cai *et al*., 2004 [13]. ABTS●+ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12–16 hr before use. The ABTS●+ solution (stable for 2 days) was diluted with water to obtain an absorbance at 734nm of 0.70±0.02. ABTS●+ solution (3ml) was added to 1 mL of test sample with various concentrations and mixed vigorously. The absorbance was measured at 734nm after standing for 6 minute. BHT was used as positive control. The ABTS●+ radical scavenging activity of the samples was expressed as $S\% = [(A_{control} - A_{sample}) / A_{control})] \times 100$, where $A_{control}$ is the absorbance of the blank control (ABTS●+ solution without test sample) and A_{sample} is the absorbance of the test sample.

2.9.5 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extractives/standard was determined by the method of Klein *et al*. [14] with a slight modification. 0.5 ml of extractives/standard at different concentrations was taken in different test tubes. 1 ml of Fe-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of 0.018% EDTA solution, 1 ml of 0.85% DMSO solution and 0.5 ml of 22% AA were added into each of the test tubes. The test tubes were capped tightly and warm at 85°C for 15 minutes into the water bath. After incubation, the test tubes were uncapped and 0.5 ml ice cold TCA (17.5%) was added to each of test tubes immediately. Three ml of nash reagent (7.5 gm of ammonium acetate, 300 µl glacial acetic acid and 200 µl acetyl acetone were mixed and made up to 100 ml) was added into all of the test tubes and incubated at RT for 15 minutes. Absorbance was taken at 412 nm wave length by UV-spectrophotometer. Percentage hydroxyl radical scavenging activity was calculated by the following equation:

$S\% = [(A_0 - A_1)/A_0)] \times 100$, where A_0 is the absorbance of the control without sample, and A_1 is the absorbance of the extractives/standard. Then percentage DPPH radical scavenging activity was plotted against concentration, and from the graph IC_{50} was calculated.

2.9.6 Lipid peroxidation inhibition assay

The lipid peroxidation inhibition assay was determined according to the method described by Liu *et al*. [15] with a slight modification. Excised rat liver was homogenized in buffer and then centrifuged to obtain liposome. 0.5 ml of supernatant, 100 µl 10 mM $FeSO_4$, 100 µl 0.1 mM AA and 0.3 ml of extractive/standard at different concentrations were mixed to make the final volume of 1 ml. The reaction mixture was incubated at 37°C for 20 minutes. One ml of (28%) TCA and 1.5 ml of (1%) TBA was added immediately after heating. Finally, the reaction mixture was again heated at 100°C for 15 minutes and cooled at RT. After cooling, the absorbance was taken at 532 nm. Percentage inhibition of lipid peroxidation was calculated by the following equation:

$S\% = [(A_0 - A_1)/A_0)] \times 100$, where A_0 is the absorbance of the control without sample, and A_1 is the absorbance of the extractives/standard. Then percentage DPPH radical scavenging activity was plotted against concentration, and from the graph IC_{50} was calculated.

3. Results

The preliminary phytochemical screening tests revealed the presence of polyphenols, terpenoids, steroids, saponins, tannin, flavonoids, alkaloids and glycosides in almost each extract of garlic which were presented in Table 1.

The total phenolic, flavonoid, flavonol and proanthocyanidin content of the different solvent extract of local variety garlic were ranged from 110.76±1.9 to 24.81±1.5 mg of GAE/gm of dry extract, 43.32±2.7 to 14.39±2.2 mg of CAE/gm of dry extract, 15.31±2.3 to 5.81±0.5 mg of QUE/gm of dry extract, 8.54±0.5 to 3.00±0.3 mg CAE/gm of dry extract, respectively (Table 2). Acetone extract showed the highest content for each compound whereas the smallest amount was observed in petroleum ether extract.

The extracts were found to increase the total antioxidant capacity and the reducing power with the increasing concentration of the extracts (Figure 1, Figure 2). Acetone extract showed the highest activity than other extracts and is significantly higher than that of BHT.

Results of DPPH, ABTS and Hydroxyl radical scavenging assay, and lipid peroxidation assay were expressed as IC_{50} (50% inhibitory concentration) which were shown in Table 3. Each extract strongly scavenged the DPPH free radical with the IC_{50} values of 7.2±0.5µg/ml (MEG), 7.8±0.85µg/ml (EEG), 5.1±0.95µg/ml (AEG), 18.5±0.65µg/ml (CEG), 19.9±0.85µg/ml (PEG). BHT (standard) showed an IC_{50} value of 12.6±0.8 µg/ml which is significantly higher than the values of AEG, MEG and EEG. The lower the IC_{50} value, the higher is the antioxidant activity.

In ABTS assay the IC_{50} values were ranged from 11.3±0.2 µg/ml (AEG) to 47.6±0.665µg/ml (CEG).

Garlic extracts were effective in scavenging the hydroxyl radical with the IC_{50} values (in µg/ml) of 16.3±0.8 (MEG), 16.7±0.8 (EEG), 15.7±0.6 (AEG), 38.4±0.8 (CEG) and 50.1±0.7 (PEG) whereas BHT showed an IC_{50} value of 20.8±0.9 µg/ml.

In lipid peroxidation inhibition assay the IC_{50} values of MEG, EEG, AEG, CEG, PEG and BHT were 22.5±0.9, 21.7±0.8, 19.5±0.3, 77.6±0.7, 91.1±0.9 and 29.1±0.9 µg/ml, respectively.

Strong positive correlation (r= 0.991) was found between antioxidant activity and phenolic content of each extract.

Table 1: Phytochemical profile of extracts from local variety garlic of Bangladesh

Extracts	P	T	St	S	Ta	F	Al	Gl
MEG	+	+	+	+	+	+	+	+
EEG	+	+	+	+	+	+	+	+
AEG	+	+	+	+	+	+	+	+
CEG	+	+	+	+	-	+	+	-
PEG	+	+	-	+	+	+	+	-

The sign (+) indicates the presence of the compounds and (-) the absence. P: Polyphenols; T: Terpenoids; St: Steroids; S: Saponins; Ta: Tannin; F: Flavonoids; Al: Alkaloids; Gl: Glycosides.

Table 2: Phytochemical content of different extracts from local variety garlic of Bangladesh

Extracts	MEG	EEG	AEG	CEG	PEG
Phenol[a]	29.72±0.9	24.81±1.5	110.76±1.9	41.19±1.3	25.65±1.1
Flavonoid[b]	20.18±2.2	22.51±1.8	43.32±2.7	25.7±2.2	14.39±2.2
Flavonol[c]	11.92±1.6	12.92±0.9	15.31±2.3	9.00±1.3	5.81±0.5
Proanthocyanidin[b]	5.17±0.5	5.13±0.4	8.54±0.5	3.62±0.4	3.00±0.3

Each value is the average of three analyses ± standard deviation. a, b and c expressed in terms of GA, CA and QU, respectively (mg of GA, CA and QU/g of dry extract, respectively).

Table 3: IC_{50} values of different extracts from local variety garlic of Bangladesh

Extracts	IC_{50} (µg/ml)			
	DPPH assay	ABTS assay	HO•	LPI
MEG	7.2±0.5	15.9±0.9	16.3±0.8	22.5±0.9
EEG	7.8±0.8	13.6±0.5	16.7±0.8	21.7±0.8
AEG	5.1±0.9	11.3±0.2	15.7±0.6	19.5±0.3
CEG	18.5±0.6	47.6±0.6	38.4±0.8	77.6±0.7
PEG	19.9±0.8	27.9±0.8	50.1±0.7	91.1±0.9
BHT	12.6±0.8	20.2±0.7	20.8±0.9	29.1±0.9

HO•: Hydroxyl radical scavenging assay; LPI: Lipid peroxidation inhibition assay

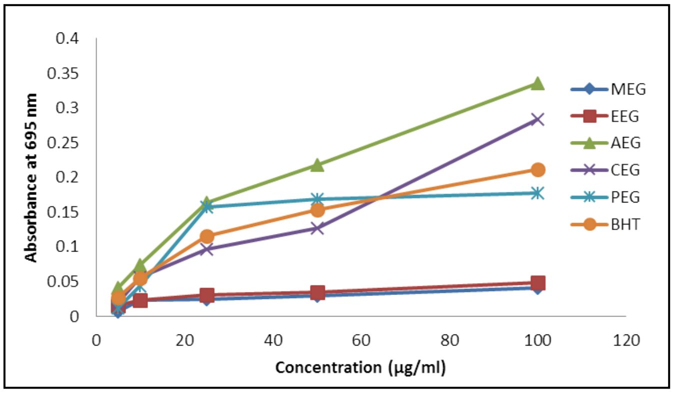

Fig 1: Total antioxidant capacity of extracts from local variety garlic of Bangladesh

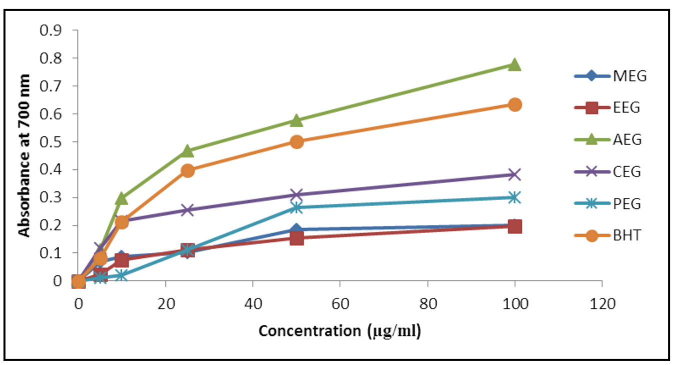

Fig 2: Ferric reducing antioxidant power of extracts from local variety garlic of Bangladesh

4. Discussion

Phytochemicals are the chemical constituents in plants with diverse biological activities such as anti-inflammatory, antioxidant, anticancer, and antimicrobial properties etc. Phenolics and flavonoids are the common antioxidants found in plant kingdom [16]. Proanthocyanidins play an important role in the prevention of various diseases, like atherosclerosis, gastric ulcer, large bowel cancer, cataracts and diabetes. Flavonols also have significant contribution to cardiovascular health [2]. Previous studies have shown that garlic is rich in polyphenol compounds. In this investigation, good amount of total phenolic, flavonoid, flavonol and proanthocyanidin has been found in local variety garlic extracts which is significantly higher than the results of the previous studies conducted on different varieties [17, 18].

Several studies reported that the differences in polyphenol content could be attributable to biological factors (genotype, cultivars), as well as environmental (temperature, salinity, water stress and light intensity) conditions. Moreover, the extraction of phenolic compounds depends on the type of solvent used, the degree of polymerization of phenolics, and their interaction [19-21]. In this report, it was confirmed that among all the employed organic solvents, acetone was the most effective for the extraction of polyphenol from garlic.

Recently, polyphenolic compounds derived from plant species have attracted particular attention due to their potentiality to reduce free radical induced tissue injury. A number of studies have demonstrated that garlic polyphenols are effective scavengers of free radicals and reactive oxygen species [17, 18, 22]. The present study has used several *in vitro* techniques to evaluate the antioxidant activity of garlic extracts. The total antioxidant capacity test (TAC) is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acidic pH. TAC of the phosphomolybdenum model evaluates both water-soluble and fat-soluble antioxidant capacity. In ferric reducing antioxidant power assay (FRAP), ferric-ferricyanide complex is reduced to the ferrous form due to the presence of antioxidants in the plant extracts. Therefore, the concentration of Fe^{2+} was monitored by measuring the formation of Perl's Prussian blue at 700nm [23]. In both TAC and FRAP assay, each extract showed increasing activity with the increase of concentration where acetone extract showed higher antioxidant activity than the others.

DPPH• (2, 2-diphenyl-1-picrylhydrazyl), a stable free radical, is widely used to study radical scavenging activities of extracts and pure compounds. In the presence of a free radical scavenger DPPH solution is decolorized from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract [24]. The ABTS radical is also commonly used to measure the radical scavenging activity of hydrogen donating and chain breaking antioxidants in many plant extracts [25]. Biochemical reaction may generate hydroxyl radical which have the ability to interact with DNA directly and therefore contribute to the cancer development. Therefore, it is essential to scavenge hydroxyl radical from our body [2]. In the present study, all of the extracts showed strong radical (DPPH, ABTS and Hydroxyl) scavenging activity in which acetone extract was proved to be the best one.

Lipid peroxidation is a chain reaction in which reactive oxygen species induce membrane damage by per oxidizing lipid moiety, specially the polyunsaturated fatty acids. Increased level of lipid peroxidation has been reported in cancer [26, 27]. In our present study, all of the extracts significantly inhibited the *in vitro* lipid peroxidation. Antioxidant activities of the present study were also found to be higher than that of previous results obtained from different garlic varieties [17, 18, 22]

It has been reported that total phenolic and flavonoid contents are highly correlated with radical scavenging and antioxidant activity of plant extract [28]. In the present study, antioxidant activity was found to increase proportionally with the increase of polyphenolic contents. The acetone extract contained relatively higher amounts of phenolics and flavonoid which may be responsible for higher radical scavenging activity compared to other extracts.

5. Conclusion

The present study suggested that local variety garlic of Bangladesh possesses a wide variety of pharmacologically important compounds and could have great importance as therapeutic agent in the protection against oxidative stress. The study also recommended that acetone would be the preferable solvent for extracting polyphenols from garlic. Further studies are needed to identify the chemical constituents of garlic responsible for antioxidant activity.

6. Acknowledgements

The authors thank all the member of the Laboratory of Clinical Biochemistry and Nutritional Sciences, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh for their constant support throughout this research.

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